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ISOLATION AND CHARACTERIZATION OF CHICK EPIPHYSEAL CARTILAGE
MATRIX VESICLE PROTEOLIPID

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Membrane dependent calcification in bacteria involves dicyclohexylcarbodiimide sensitive, ion translocating proteolipids. Since epiphyseal cartilage matrix vesicles appear to support calcification by a similar mechanism, this study was undertaken to determine the chemical composition and dicyclohexylcarbodiimide binding characteristics of matrix vesicle proteolipids. Matrix vesicles were prepared from the epiphyseal plate growth cartilages of six to eight

week old broiler strain chickens. Presence of matrix vesicles was confirmed by transmission electron microscopy. Specific activities of alkaline phosphatase and 5'-nucleotidase were enriched in the matrix vesicles; ouabain sensitive Na^+/K^+ adenosine triphosphatase was enriched in the plasma membranes. Matrix vesicle proteolipid was isolated by organic solvent extraction with either $\text{CHCl}_3:\text{CH}_3\text{OH}$ at 2:1 (proteolipid fraction 1) or $\text{CHCl}_3:\text{CH}_3\text{OH}:\text{HCl}(\text{conc})$ at 200:100:1 (proteolipid fraction 2), followed by Sephadex LH-20 column chromatography. Proteolipid protein composition and ^{14}C -dicyclohexylcarbodiimide binding characteristics were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis and autoradiography. Proteolipid fraction 1 contained a single W-shaped band at 6,000-8,000 M_r while proteolipid fraction 2 exhibited bands at 4,000-5,000 M_r and 14,000-16,000 M_r . Protein species at 6,000-8,000 and 4,000-5,000 M_r bound ^{14}C -dicyclohexylcarbodiimide in a dose dependent manner. Competitive binding between ^{14}C -dicyclohexylcarbodiimide, the hydrophilic carbodiimide ethyldimethylaminopropylcarbodiimide or glycine ethyl ester, to the proteolipid, suggests that ^{14}C -dicyclohexylcarbodiimide binds specifically to carboxyl groups in hydrophobic regions of the protein. These results indicate that the protein species of matrix vesicle proteolipids differ in molecular weight from those isolated from

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The University of Texas Graduate School of Biomedical
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in Partial Fulfillment
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By
Terrell Cohen D.D.S., B.S.

San Antonio, Texas

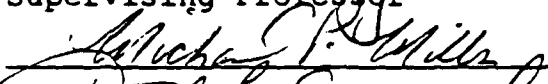
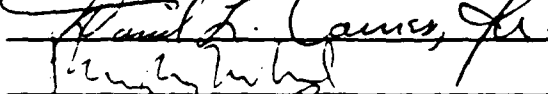
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ISOLATION AND CHARACTERIZATION OF CHICK EPIPHYSEAL CARTILAGE
MATRIX VESICLE PROTEOLIPID


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This thesis is dedicated to my parents; Chester and Bernice and to my wife and children; Cafi, Jeffrey and Tamara. Their love, support, and encouragement has made the time spent in the laboratory and at the writing desk not only possible, but worthwhile.

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calcifying bacteria. A subset of matrix vesicle proteolipids exhibit ^{14}C -dicyclohexylcarbodiimide binding characteristics comparable to ion translocating proteolipids of bacteria.

The similarities between proteolipids from calcifying bacterial and those extracted from calcifying matrix vesicles, indicate that the basic mechanisms of *de novo* mineralization may proceed by similar paths in these models.

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I. INTRODUCTION AND LITERATURE REVIEW

A. Introduction.

De novo mineralization in biological systems is a complex process whether it occurs in bacteria or in vertebrate animals. This holds true for both physiologic and pathologic deposition of mineral. In a given tissue type, mineralization depends on cellular regulation of the interaction of cellular membrane components and macromolecules that comprise the extracellular matrix. In vertebrate tissues, one site of initial hydroxyapatite formation occurs in extracellular membrane organelles called matrix vesicles. While specific events at the cellular level may vary among tissue types, there is evidence that, similarities exist in the basic mechanisms involved in the calcification process at the molecular level.

Two model systems have been useful in studying basic *de novo* mineralization mechanisms. One of these systems, a bacterial model, has allowed biochemical comparisons to be made between calcifying and non-calcifying cultures. This approach has direct significance for understanding the mechanisms of calcification involved in dental calculus formation. It has the advantages of being a relatively inexpensive, simple system, that is easily controlled in experimental protocols. In addition, the bacterial model can provide large sample sizes which facilitates experiments utilizing extracted proteolipids.

The vertebrate model uses a variety of tissues from an assortment of animals including rats, rabbits, cattle, and chickens. In this system, comparison of cellular and biochemical components between non-calcifying tissues and tissues at various stages in the mineralization process can be made. While the vertebrate model is closely related to the calcification process in human tissues, it has the disadvantages of being a complex system, yielding very small samples of matrix vesicles, which must be isolated from a collagenous matrix.

Both the bacterial and vertebrate models described are based on the idea that *de novo* mineralization is controlled by cell membranes or components derived from cell membranes. It has been hypothesized that both model systems utilize similar membrane associated macromolecules to initiate hydroxyapatite crystal formation. If this can be demonstrated, it follows that the mechanisms of mineralization that might be elucidated using the bacterial model will also apply to vertebrate tissues.

B. Association of Matrix Vesicles and Mineralization.

In 1967, two investigators (Bonucci, 1967; Anderson, 1967) independently reported that small membrane bound vesicles were associated with initial evidence of mineral formation in calcifying cartilage matrix. Under transmission electron microscopy these matrix vesicles appeared round to ovoid in shape with diameters ranging from

300A up to 2500A (Anderson, 1967; Bonucci, 1967). At high magnification the membrane structure appears to be trilaminar, corresponding to a unit membrane (Anderson, 1969). Cartilage mineralization was preceded by the formation of these vesicles in which initial crystallization appeared to form within the membrane borders. As the hydroxyapatite crystals enlarged, ruptured vesicles were left behind (Anderson, 1967; Bonucci, 1967).

Since these initial discoveries, it has been demonstrated that matrix vesicles are associated with deposition of calcium-phosphate mineral complexes in a number of tissues including cartilage (Ali et al., 1977; Ali, 1983; Anderson, 1969; Anderson, 1967; Bonucci, 1967; Howell et al., 1976; Anderson et al., 1981), bone (Anderson, 1973; Bernard and Pease, 1969; Bonucci, 1971; Gay, 1977), dentin (Bernard, 1972; Eismann and Glick, 1972; Siska and Provenza, 1972; Slavkin et al., 1972), and cementum (Hayashi, 1985). Matrix vesicles have been implicated in mineralized tissue repair processes with their discovery in fracture callus (Ketenjian and Arsenis, 1975; Boskey et al., 1980), and in reparative dentin (Bab et al., 1982). In addition, matrix vesicles appear to be involved in numerous pathologic calcification processes including atherosclerosis (Tanimura et al., 1983), aortic valve calcification (Kim, 1976), osteoarthritis (Ali, 1977), osteosarcoma (Muhlrad et al., 1978), non-skeletal neoplasms (Anderson et al., 1969),

and renal calcifications (Ganote et al., 1975). Formation of dental calculus (Boyan et al., 1982; Ennever et al., 1978B; Sidaway, 1980) and calcification on failed intrauterine devices (Gonzales, 1981) represent examples of microbial calcification which have features in common with matrix vesicle calcification.

It is hypothesized that matrix vesicles control the biochemical environment to regulate mineralization in both time and space. This is supported by the observations that initial hydroxyapatite formation occurs on the inner surface of the membrane - an enclosed environment - in the hypertrophic zone of cartilage (Ali, 1976; Ali et al., 1978; Anderson, 1969), in dentin (Bernard, 1972), and in the fracture callus (Lowe et al., 1983). The findings that matrix vesicles are truly extracellular (Anderson et al., 1970; Glauert and Mayo, 1977), and are oriented in tissues in a non-random fashion (Reinholt et al., 1982), strengthen the concept that they also modulate the spatial aspect of mineralization.

C. Origin of Matrix Vesicles.

Although there is controversy on the origin of matrix vesicles, three of the four current theories on their biogenesis propose a cellular origin (Rabinovitch et al., 1976). This suggests that *de novo* mineralization associated with matrix vesicles is indeed under cellular control. The fourth theory, that matrix vesicles arise from the

interaction of extracellular macromolecules, has little supporting evidence. These theories are described in detail below.

It is likely that the majority of matrix vesicles are derived by budding off of cell membranes and/or from cell degeneration. These theories are not mutually exclusive. The budding or blebbing theory originally proposed by Anderson (1969) states that matrix vesicles arise from intact cell membranes. This hypothesis has been supported by electron micrographs utilizing freeze fracture and freeze etching techniques (Borg et al., 1978; Cecil and Anderson, 1978), and by transmission electron microscopy of very thin sections of cartilage examined stereoscopically (Anderson, 1978; Glauert and Mayo, 1977). The phospholipid composition and arrangement suggests that matrix vesicles may arise from nearby cells (Wuthier, 1976; Wuthier et al., 1977; Hale and Wuthier, 1987). Additional support for the blebbing hypothesis comes from cell culture studies of chondrocytes which indicate that matrix vesicles are formed without disruption of cell membranes (Glaser and Conrad, 1981). The phospholipid composition of vesicles formed in culture is similar to vesicles formed *in vivo*. Like the vesicles isolated from intact tissues, the phospholipid composition of the matrix vesicles produced in cell cultures is enriched in phosphatidylserine.

Evidence that matrix vesicles can arise from cell degeneration comes from observations of matrix vesicles associated with cell degeneration in pathologic calcifications: arteriosclerosis (Kim, 1982; Tanimura et al., 1983), tumors (Kim, 1982), and rickets (Reinholt et al., 1984). Another line of support for this theory is that matrix vesicles increase in number in aging cartilage tissue (Bonucci and Dearden, 1976).

Biochemical analyses provide further evidence for both the budding and cell degeneration hypotheses. A variety of plasma membrane marker enzymes such as alkaline phosphatase (Ali et al., 1970; Anderson et al., 1970; Matsazuwa and Anderson, 1971; Muhlrad et al., 1983; Hsu et al., 1985), ouabain sensitive Na^+/K^+ ATPase (Ali et al., 1970; Matsazuwa and Anderson, 1971; Kanabe et al., 1976; Glaser and Conrad, 1981), inorganic pyrophosphatase (Felix and Fleisch, 1976), 5'-nucleotidase (Ali et al., 1970), and nucleoside triphosphate pyrophosphohydrolase (Hsu, 1983; Siegel et al., 1983; Caswell et al., 1987) are present in matrix vesicles. Matrix vesicles have lipid profiles characteristic of plasma membranes: they contain high levels of sphingomyelin, have a high cholesterol:phospholipid ratio (Peress et al., 1974; Wuthier, 1975), and they are enriched in phosphatidylserine (Cotomore et al., 1971). However, the percent phospholipid composition is distinct from that of the plasma membrane in that phosphatidylethanolamine content is lower and

phosphatidylserine content is higher in the matrix vesicles (Peress and Anderson, 1974).

Formation by extrusion of preformed intracytoplasmic vesicles through the cell membrane, while theoretically possible, is not considered to be a major source of matrix vesicles. This hypothesis was first suggested by Slavkin (1972) in his study of matrix vesicle formation associated with odontoblasts. Evidence that a subset of matrix vesicles represents extruded lysosomes is based on cytochemical demonstration of acid phosphatase and aryl sulfatase in some vesicles (Thyberg and Friberg, 1972), but has not been confirmed (Ali et al., 1970; Matsazuwa and Anderson, 1971). Rabinovitch and Anderson (1976) have suggested that matrix vesicles may arise by extracellular assembly of macromolecular subunits. Although this theory has little experimental support, it has been demonstrated that matrix vesicle preparations dissolved with detergents were able to reform into calcifiable liposomes (Hsu and Anderson, 1978) indicating that the mechanism is possible.

D. Mechanisms of Mineralization.

Chemical (Wuthier and Gore, 1977) and electron probe analysis (Ali, 1976) has demonstrated that matrix vesicles are enriched in calcium, and are also associated with solid phase calcium phosphate mineral (Ali, 1976; Ali et al., 1977; Gay et al., 1978; Barckhaus et al., 1981; Ozawa and Yamamoto, 1983; Morris et al., 1983); Anderson (1984) has

proposed a two stage theory for the mechanism of *de novo* mineral formation by matrix vesicles. Phase I is the intravesicular initiation of mineralization while phase II is concerned with mineral deposition and hydroxyapatite crystal growth outside of the matrix vesicle membrane.

1. De novo membrane-associated mineralization.

Phase I begins with attraction of calcium ions to acidic phospholipids which are concentrated in matrix vesicle membranes (Peress et al., 1974; Wuthier, 1975). The inner leaflet of the membrane may provide an ideal environment for the initiation and growth of mineral deposits because it selectively localizes the calcium attracting phospholipid phosphatidylserine (Majeska and Wuthier, 1976; Irving, 1976). Yaari and Shapiro (1982) demonstrated that phosphatidylserine, when dissolved in an organic solvent, can transport Ca^{+2} from one aqueous compartment to another. This transport is promoted by a pH gradient across the organic solvent and by the presence of phosphate.

These observations suggest that phosphatidylserine may act as an ionophore transporting Ca^{+2} and phosphate to the interior of the matrix vesicle via the formation of specific phospholipid:calcium:phosphate complexes (CPLX) (Yaari et al., 1984). Calcium accumulates ahead of phosphate (Ali, 1976; Ali et al., 1977), which is concentrated locally by phosphatases (Matsazuwa and Anderson, 1971; Sajdera et al.,

1976; Kanabe et al., 1976). This may be due in part to alkaline phosphatase acting across the matrix vesicle membrane to elevate the concentration of P_i internally (Cyboron et al., 1982; Kanabe et al., 1976). When concentrations of Ca^{+2} and P_i within the enclosed environment of the matrix vesicle reach a critical level, an amorphous mineral complex forms, either as amorphous calcium-phosphate (Wuthier et al., 1977; Gay et al., 1978), or as a poorly defined crystalline structure (Brown, 1966; Roufosse et al., 1979).

2. Matrix vesicle degeneration.

Phase I of the mineralization mechanism ends when initial mineral deposits become exposed to the extravascular environment due to breakdown of the matrix vesicle membrane. Early electron microscopic studies of EDTA decalcified tissues revealed remnants of matrix vesicles indicating that they had degenerated during the calcification process (Anderson, 1969). Morphometric studies indicate that vesicles decrease in number as calcification proceeds (Menanteau et al., 1982). The exact mechanism of matrix vesicle degeneration is not clear, but may involve two steps. Partial degradation of the matrix vesicle membrane by a variety of enzymes including phospholipase A2 (Wuthier, 1973), peptidases (Hirschman et al., 1983), or proteases (Fujiwara et al., 1983; Katsura and Yamada, 1985), may cause leaks that allow Mg^{+2} to leak out.

Loss of this inhibiting ion would accelerate calcification by promoting conversion of amorphous mineral to apatite (Wuthier et al., 1977; Wuthier and Gore, 1977). Finally, growth of internal hydroxyapatite crystals may distort and rupture the matrix vesicle membrane (Anderson, 1984).

3. Regulation of extra-vesicular crystal growth.

Phase II refers to hydroxyapatite crystal proliferation brought about by the exposure of apatite crystals to the extra-vesicular environment. A key point is that the initial apatite crystals formed within the vesicles must be present for phase II to proceed, as the extracellular fluid under physiologic conditions does not contain sufficient concentrations of Ca^{+2} or P_i ions to initiate mineralization (Howell et al., 1968). After disruption of the matrix vesicle membrane, crystal growth rate is dependent on naturally occurring regulatory factors. Inhibition may be due to absence of Ca^{+2} and P_i ions as seen in rickets and osteomalacia (Anderson et al., 1975, Anderson et al., 1980), or may be due to the presence of natural inhibitors of calcification including: inorganic pyrophosphate (Fleisch and Bisaz, 1985), adenosine triphosphate (ATP) (Betts et al., 1975; Termine and Conn, 1976), noncollagenous phosphoproteins (Desteno and Feagin, 1975; Menanteau et al., 1982; Linde et al., 1983), or glutamic acid containing proteins (Price et al., 1976). Increased concentrations of Ca^{+2} and P_i ions enhance the

rate of mineral deposition in phase II (Dudley and Shapiro, 1961). Control of one or more of these regulating factors could allow regulation of the entire calcification cascade.

E. Phospholipid:calcium:phosphate complexes and proteolipids.

1. Role of phospholipid:calcium:phosphate complexes.

In addition to raising the local concentrations of Ca^{+2} and P_i ions the components of the matrix vesicle membrane may also bring the ions together in a spatial relationship that promotes calcification. Phosphatidylserine and perhaps other Ca^{+2} attracting phospholipids may act as a trap to locally elevate Ca^{+2} concentrations on the inner leaflet of the membrane. The result is high concentrations of Ca^{+2} and P_i combining with membrane lipid to form phospholipid:calcium:phosphate complexes (CPLX). CPLX is present in matrix vesicles (Wuthier and Gore, 1977) and is associated with both physiologic and pathologic calcification processes (Boskey and Posner, 1976, 1977; Boskey et al., 1977; Boskey et al., 1982). In metastable calcium phosphate solutions, CPLX has the ability to initiate hydroxyapatite formation (Boskey and Posner, 1977).

2. Calcifiable proteolipids.

A class of compounds known as proteolipids are also able to initiate hydroxyapatite formation in metastable calcium phosphate solutions (Ennever et al., 1977; Ennever

et al., 1978B; Ennever et al., 1980). Proteolipids are a class of hydrophobic proteins that are readily soluble in organic solvents (Folch and Stoffyn, 1972), but soluble in aqueous solvents only with great difficulty (Folch and Sakura, 1976). They are often complexed with lipid as part of their primary structure and tend to be strongly associated with the acidic phospholipid phosphatidylserine, which has been shown to be implicated in concentration of Ca^{+2} , and phosphatidylinositides (Schlesinger, 1981; Stoffyn and Folch, 1971). Calcifying proteolipids refer to those proteolipids that precipitate hydroxyapatite in bicarbonate buffered metastable calcium phosphate solutions. They have been identified in bone (Ennever et al., 1977), chicken epiphyseal cartilage (Boyan et al., 1983), dentin (Bonucci, 1971), atherosclerotic plaque (Ennever et al., 1980), calcifying oral bacteria (Boyan-Salyers et al., 1978), calculus (Vogel, 1971; Ennever et al., 1976; Ennever et al., 1978B; Boyan et al., 1982; Boyan et al., 1983), and submandibular salivary gland stones (Boskey et al., 1981).

3. Association of CPLX and proteolipid.

Calcifiable proteolipids are implicated in membrane associated mineral deposition and are related to the CPLX complex (Shapiro, 1970; Vogel, 1971; Vogel et al., 1976; Ennever et al., 1976; Ennever et al., 1977; Ennever et al., 1978A; Vogel et al., 1978; Boyan et al., 1982). CPLX is formed by proteolipids prior to hydroxyapatite deposition

which implies that the interaction of calcifiable proteolipids with phospholipids, Ca^{+2} , and P_i is an initial step in the mineralization process. CPLX and proteolipids have been co-isolated from bacteria (Boyan et al., 1982; Boyan and Boskey et al., 1984), and chicken bone (Koniuta et al., 1984). Using a rabbit model system, Raggio et al. (1986) demonstrated that proteolipids and CPLX isolated from rabbit bone are capable of inducing hydroxyapatite formation *in vivo* when implanted in rabbit mesenchyme. Under these experimental conditions, the proteolipids supported CPLX formation.

F. Current Concepts of Calcifiable Proteolipid Functions.

1. Experimental approaches.

Experimental approaches to the role of proteolipids in calcification are based on the assumption that the mechanisms involved are independent of the organisms and tissues. Two model systems have been used to test various aspects of the hypothesis that proteolipid dependent calcification is regulated by the concentration of the proteolipid(s) themselves, the phospholipid environment, and the availability of mineral ions. The bacterial model uses calcifying strains of *Bacterionema matruchotti*. Alteration of the culture media allows manipulation of the mineralization process without any interference from a collagenous matrix. The vertebrate model analyzes matrix

vesicle components extracted from normal or rachitic animals. It has been shown in both models that only lipids associated with proteolipids are capable of inducing CPLX and hydroxyapatite formation, and the ability of the crude phospholipids to initiate calcification depends on a single proteolipid fraction obtained on Sephadex LH-20 chromatography (Ennever et al., 1976; Boyan-Salyers, 1978B).

2. Proteolipids and membrane structure.

Evidence that proteolipids play a role in the structural organization of lipid membranes (Boggs et al., 1977) have been substantiated in experiments with rachitic rats and chickens. Alteration of proteolipid composition in vitamin D dependent rickets has been demonstrated (Boyan and Ritter, 1984). Proteolipids isolated from rachitic cartilage fail to support *in vitro* hydroxyapatite formation. In addition there is an increase in phospholipid content, particularly of those lipids not associated with CPLX or hydroxyapatite formation. Boskey and Weintraub (1984) have also demonstrated that the phospholipid and CPLX content of rachitic tissues is altered by treatment with vitamin D. It is possible that changes in membrane fluidity due to altered arrangement of phosphatidylserine and phosphatidylinositides around the core proteolipids could alter CPLX formation. Decreased mineralization in rachitic animals would then be due to insufficient quantities of Ca^{+2} and P_i secondary to non-functional CPLX (Koniuta et al., 1984; Boyan, 1985).

Use of x-ray diffraction, mineral ratios, electron microscopy, and biochemical analysis of proteolipid, lipid, and protein composition with the bacterial model has at least partially defined the sequence of mineral deposition. Proteolipid concentration and the ratio of proteolipid to associated phospholipid increase with time and peak in 8 day old cultures. Coinciding with this is the first crystallographic evidence of hydroxyapatite formation and the resolution of four sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) bands, seen at 6 days, into a single band of M_r 10,000 at day 8 (Boyan-Salyers, 1978A; Landis and Glimcher, 1982; Boyan et al., 1982; Boyan et al., 1984).

3. DCCD binding characteristics of proteolipids.

Recent investigations into the roles of proteolipids in initiation of *de novo* hydroxyapatite formation are based on known functions of proteolipids from non-calcifying tissues where they form an integral portion of plasma membranes (Fillingame, 1980). These proteolipids may function as ionophores which permit the passage of charged particles across lipid membranes (Senior and Wise, 1983). One example of this is the F_0 component of mitochondrial ATP synthetase. F_0 is a proton translocating ionophore which exhibits a predominant protein species at M_r 10,000, specifically binds N,N'-dicyclohexylcarbodiimide (DCCD), and loses its ability to act as an ionophore when it

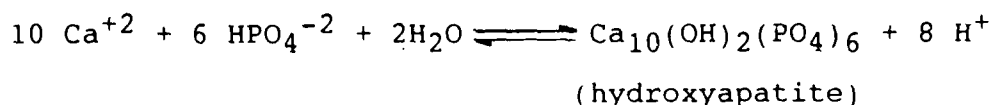
has bound DCCD (Cattell et al., 1971; Fillingame, 1976; Sone et al., 1978; Sebald et al., 1980; Fillingame, 1980; Lin and Lees, 1982). Similar DCCD binding properties have been shown to exist in ATPase from bacteria (Negrin et al., 1980; Perlin et al., 1983), sarcoplasmic reticulum (Murphy, 1981), and chloroplasts (Nelson et al., 1977).

Recent studies using the bacterial model indicate that the proteolipid composition is more complex than originally thought (Deresewski and Boyan, 1982). The 10,000 M_r SDS-PAGE band associated with hydroxyapatite formation in culture was based on proteolipids extracted using chloroform:methanol at 2:1 (v/v). Use of an acidified organic solvent, chloroform:methanol:HCl(conc) at 200:100:1 (v/v/v), results in the resolution of 5 bands by SDS-PAGE at M_r 8,500, 10,000, 24,000, 38,000, 45,000, 65,000, 85,000 (Boyan et al., 1987; Swain and Boyan, 1987; Swain et al. unpublished data). If the neutral and acid solvent extractions of proteolipids are performed sequentially, then the 10,000 M_r band predominates in the neutral proteolipid extract, but is absent in the acid proteolipid extract. Only the 8,500 M_r species binds DCCD. *In vitro* studies indicate that the 10,000 M_r protein can support hydroxyapatite crystal formation, but both the 8,500 and 10,000 M_r proteolipid species are required for function as an ionophore. Ionophoric uncoupling of proton transport across liposome membranes depends on the combined

incorporation of both acid and neutral proteolipid extracts, and is abolished by addition of DCCD (Boyan et al., 1985; Swain and Boyan, 1987).

G. Model for Membrane Associated Proteolipid Calcification.

These data suggest a model for *in vivo* hydroxyapatite formation in a closed membrane system of either bacterial or matrix vesicle origin. The 10,000 Mr protein predominates on the inner leaflet of the membrane where it acts to promote hydroxyapatite crystal formation. The combination of the 8,500 and 10,000 Mr protein species span the membrane and together act to transfer protons from the internal vesicle environment to the outside. When the overall stoichiometry of the reaction that culminates in



hydroxyapatite formation is considered (Wadkins et al., 1974), the importance of proton transport becomes apparent. A mechanism to remove accumulating hydrogen ions is necessary in order to maintain the pH of the internal environment at a level that will allow the reaction to proceed to the right (Campbell and Speeg, 1969). Alkaline phosphatase may play a role in calcification, not by acting as a phosphate transferase (Register et al., 1984; Register and Wuthier, 1984; Wuthier et al., 1985; Register et al.,

1986), but by acting on phosphatidylethanolamine to alter membrane fluidity (Boyan et al., 1987), or by removing inhibitors of mineral crystal growth (Register and Wuthier, 1985).

H. Statement of Problem.

The composition and DCCD binding characteristics of *Bacterionema matruchotti* proteolipid are well established (Boyan et al., 1985). However, there are no reports in the literature that describe the properties of proteolipids associated with calcifying tissues of vertebrate animals. In order to extend the proposed role of proteolipids elucidated in the bacterial model to vertebrate systems, it is necessary to study the properties of proteolipids extracted from matrix vesicles of calcifying tissues in order to find out if they have similar properties. The goal of this research is to characterize the DCCD binding characteristics of the proteolipid specie(s) associated with chicken epiphyseal cartilage matrix vesicles.

II. MATERIALS AND METHODS

A. Isolation of Matrix Vesicles.

1. Dissection Technique.

Immediately after slaughter, 200-500 feet from broiler strain chickens (49-52 days old, Holly Farms, Seguin, Texas) were collected and placed on ice. Epiphyseal growth plate cartilages of the three proximal articulating surfaces of the metatarsal bone (Sisson, 1953) were removed by sharp dissection (plates 1-3) and placed into chilled 50 mM TRIS-HCl buffer, 0.25M dextrose, pH 7.4. Calcified portions of the epiphyses were eliminated from the sample by careful inspection. Excess blood and debris were removed by three consecutive rinses in buffer. The sample was drained without vacuum and minced to approximately 1 mm³ pieces by sharp dissection. After determination of the wet weight, the sample was divided into portions weighing approximately 15 grams each.

2. Isolation of matrix vesicles.

Collagenase digestion was used to break down the connective tissue matrix in order to release the matrix vesicles. This was accomplished by adding 15 ml of filtered (Whatman 50H paper) 0.03% Type II collagenase (Worthington Biochemical Corp, Freehold, NJ, catalog number 4176, lot # 47S266) in 50 mM Tris, 0.25 M dextrose, pH 7.4, per gram of

cartilage. Digestion was carried out at 37°C for 15 hours using gentle agitation (Orbit model 35-35 automatic shaker at 130 rpm). The digest was filtered through #45 nylon mesh without vacuum. Matrix vesicles were isolated as described by Ali et al. (1976). Remaining solid material was discarded and the filtrate was centrifuged at 750 X g for 5 minutes to pellet whole cells, cell fragments, and organelles. The pellet was saved for preparation of the cell membrane fraction. The resulting supernatant was centrifuged at 13,000 X g for 10 minutes to pellet mitochondria and debris which was discarded. The supernatant was centrifuged at 85,000 X g for 60 minutes to yield a matrix vesicle enriched pellet. The supernatant was carefully poured off and discarded; the pellet was suspended with gentle mixing in 4.3 ml of 0.9% NaCl. 100 ul was set aside for examination by transmission electron microscopy and the remainder stored at -70°C.

B. Plasma Membrane Isolation.

Plasma membranes were isolated using the method described by Fitzpatrick et al. (1969) for renal plasma membranes. The pellet containing whole cells, fragments, and organelles was suspended in 5ml of 2M sucrose, homogenized, and centrifuged at 14,460 X g for 10 minutes. The pellet was discarded. The supernatant was diluted with cold ultrapure H₂O to yield a 0.25M sucrose solution and centrifuged at 39,000 X g for 10 minutes. The supernatant

If one or both of the top sampling packages are unacceptable (TR1: +/-), but both TRs in locations TR2 are acceptable (TR2: ++), then all packages in the lot but the top layer are saved.

Additional packages of food can be considered as saved by taking into account the protective insulating effect of other lots. Consequently, treat as a large unit two or more lots that are in contact or in an extremely close proximity to each other.

B. Mechanical Failure of Horizontal Display Cabinets

1. Thick Packages

Refer to Fig 4, A for the specific sampling sites. Keep in mind that the sampling sites are in duplicate for each lot. There are five sampling sites (TR1, TR2, TR3, TR4, and TR5) for lots with three or more layers. For lots of one and two layers, there are two and three sampling sites, respectively. As described previously, the thermometer should penetrate each site such that the tip is no more than one inch from the top and the two sides of the food sample.

Refer to Table 3 to determine the scenarios and appropriate decisions for various food lots. Scenarios 1 to 6 are primarily for lots of three or more layers. However, scenarios 1, 3, and 4 are also applicable to two-layered lots while for one-layered lots, scenarios 1 and 3 are applicable. Scenarios 7 and 8 are applicable only for two-layered lots and scenario 8 is applicable only for one-layered lots.

TABLE 3. Scenarios of temperature readings (TRs) of selected portions of a food lot of thick packages and subsequent decisions using the simplified form sampling strategy^a

Scenarios ^b	TRs					Decisions
	TR1	TR2	TR3	TR4	TR5	
1	++	++	ND ^c (++) ^d	++	++	Save whole lot
2	++	++	ND(++/-) ^d	++	++	Reject bottom layer
3	+-/-	++	ND(++) ^d	++	++	Reject top layer
4	+-/-	+-/-	++	++	++	Reject top two layers
5	+-/-	+-/-	++	++	+-/-	Reject top two layers and bottom layer
6	+-/-	+-/-	+-/-	ND	ND	Reject whole lot
7	++	+-/-	ND			Reject lot
8	++	+-/-				Reject lot

^a"++" indicates that two samples for TRs did not exceed acceptable temperature limit; "+-/-" indicates that a single TR result is above the temperature limit and that if the first TR result is above the temperature limit, a second TR is unnecessary.

^bScenarios 1-5 are applicable to lots consisting of three or more layers. Scenarios 1, 3 and 4 are applicable to two-layered lots; Scenarios 1 and 3 are applicable to one-layered lots.

^cND means not determined.

^dApplicable only to two-layered lots.

2. Thin Packages

Refer to Fig 4, B for the specific sampling sites. The thermometer penetration should be such that the tip is no more than one inch from the sample surface and outer corner of the food sample and equidistant from the top and bottom of the package.

Follow the sequence described in Table 4 starting with Scenario 1. All the scenarios are applicable to lots of four or more layers, with sampling sites limited to TR1 and TR2. For the three layered lot, Scenarios 1 to 4 and 6 are applicable.

If in a four layered lot, TRs 1 and 4 are acceptable, then the whole lot is saved.

If in a six layered lot, TR1 is unacceptable but TR2 and TR5 are acceptable, then only the top layer is rejected. If in addition to TR1, TR2 is also unacceptable but TR3 and TR5 are acceptable, then only the top two layers of the lot are rejected. If TRs 1, 2, and 3 are unacceptable then the whole lot is rejected.

C. VERTICAL DISPLAY CABINETS

In contrast to the complexity of TRs in food products in horizontal cabinets, the sampling strategy for vertical cabinets is much simpler. This is due to the inherent lack of insulation of open vertical cabinets; moreover, there is a low profile stacking arrangement in vertical cabinets.

Before sampling begins, the lot should be of uniform height ("A" configuration) as discussed previously for the horizontal cabinets. The simplified sampling strategy is appropriate for a food lot that has a dimension of three stacks in length by four stacks in width or less. Do a TR on the most exposed section of the top front corner packages. The entire lot is saved if the TR is +, otherwise the lot is rejected.

TABLE 4. Scenarios of temperature readings (TRs) of selected sites in a food lot of thin packages and corresponding decisions (simplified strategy).

Scenarios	TR1	TR2	TR3	TR4	TR5	Decisions
1	++	ND(++) ^a	ND(++) ^b	ND(++) ^c	++	Save whole lot
2	++	ND(++/-) ^a (++) ^b	ND(++/-) ^b (++) ^c	++(++/-) ^c	++/-	Reject bottom layer
3	++/-	++	ND(++) ^b	ND(++) ^c	++	Reject top layers
4	++/-	++/-	++	ND(++) ^c	++	Reject top two layers
5	++/-	++/-	++	++(++/-) ^c	++/-	Reject top two layers and bottom layer
6	++/-	++/-	-/-	ND	ND	Reject whole lot

^aApplicable only to two-layered lots.

^bApplicable only to three-layered lots.

^cApplicable only to four-layered lots.

ND means Not determined

VII. TEMPERATURE READINGS AND DECISION MAKING: LONG FORM SAMPLING STRATEGY

A. Power Failure of Horizontal Display Cabinets

This is a more discriminating sampling strategy. The two corner sampling stacks (see darkened stacks in Figure 5) are designated to represent the S packages which are more exposed and are therefore warmer than the C packages; the latter are represented by one sampling C stack. Thick and thin packages are treated alike when power failure occurs.

If the top sampling S packages are unacceptable but the second to the top sampling S packages and the top sampling C packages are acceptable, then only the top S packages are rejected. With the simplified sampling strategy, the whole top layer would have been automatically rejected.

The long form sampling strategy is particularly advantageous when there is a good chance to save a large number of C packages. For instance, if the temperatures of the S packages of a lot are a few degrees above specification, it is likely that the C packages are within specification and are therefore acceptable. In essence, the long form strategy samples food lots not only by layers but also by stacks.

Additional savings can be achieved by taking into account the protective insulating effect of other lots. Consequently, treat as a large unit two or more lots that are in close proximity of each other. As an illustration, Lot A in Figure 6 has one side in contact with Lot B. So in essence, the S stacks of the protected side of Lot A are considered as C stacks except those corner stacks which remain as the outer portion of the larger unit. In the case of Lot B whose two sides are protected by the adjacent lots, A and C, with the exception of the corner stacks, the shielded S packages of both sides of the lot are considered as C stacks.

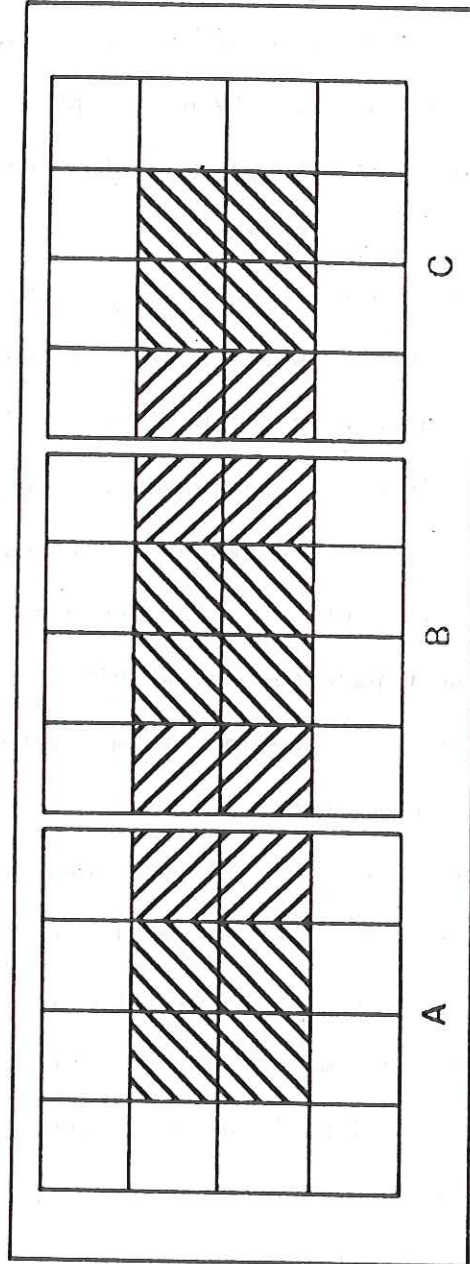


Figure 6. Schematic representation of the food lot situation in a horizontal display cabinet (top view) in regard to the heat shield effect of neighboring food lots. The core stacks are shown as shaded squares.

B. Mechanical Failure of Horizontal Display Cabinets

1. Thick Packages

The sampling strategy is similar to the simplified form except that in addition to the two corner sampling stacks that now represent only the S packages, there is also a stack that represents the C packages. Follow the applicable scenarios as shown in Table 5.

As an illustration, if a refrigeration failure should occur as indicated in scenario 4, the use of a simplified sampling strategy would reject the top two layers.

2. Thin Packages

This sampling strategy attempts to save additional temperature-abused foods by differentiating the S packages from the C packages. Thus, Table 6 is simply an expanded version of the simplified sampling strategy in Table 4 to accommodate the scenarios of the C packages. There are four subscenarios for scenario 6, differing only in the TRs of their sampling C packages. As described in a previous section, to simplify the sampling procedure lots of similar type of food and size that are next to each other can be combined and treated as one lot.

C. Vertical Display Cabinets

If the dimension of a lot is 4x4 packages or more, the long form sampling strategy is used. This involves dividing the lot into two groups. The side (S) group includes the S packages of the front and back ends and those forming the left and right perimeter of the lot. Those packages surrounded by the S packages constitute the core (C) group.

TABLE 5. Scenarios of temperature readings (TRs) of selected portion of a food lot of thick packages and subsequent decisions using the long form strategy^a

Scenarios ^b	Side(S) Core(C)	TR1	TR2	TR3	TR4	TR5	Decisions
1	S	++	++	ND ^c (++) ^c	++	++	Save all S packages
	C	ND	ND	ND	ND	ND	Save all C packages (C1)
2	S	++	++	ND ^c (+-/-) ^c	++	+/-	Reject bottom S packages
a	C	ND	ND	ND	ND	+	C1
b	C	ND	ND	ND(-) ^c	ND	-	Reject bottom C packages
3	S	+/-/-	++	ND(++) ^c	++	++	Reject top S packages
a	C	+	+	ND	ND	ND	C1
b	C	+/-/-	+	ND	ND	ND	Reject top C packages (C2)
4	S	+/-/-	+/-/-	++	++	++	Reject top two S packages
a	C	+	+	ND	ND	ND	C1
b	C	-	+	ND	ND	ND	C2
c	C	-	-	ND	ND	ND	Reject top two C packages (C3)

TABLE 5. (Continued) Scenarios of temperature readings (TRs) of selected portion of a food lot of thick packages and subsequent decisions using the long form strategy^a

Scenarios ^b	Side(S) Core(C)	TR1	TR2	TR3	TR4	TR5	Decisions
5	S	+-/-	+-/-	++	++	+-/	Reject top two S packages and bottom of S packages
a	C	+	+	ND	ND	+	C1
b	C	-	+	ND	ND	+	C2
c	C	-	-	ND	ND	+	C3
d	C	-	-	ND	ND	-	Reject top two C packages & bottom C packages (C4)
6	S	+-/-	+-/-	+-/-	ND	ND	Reject S package
a	C	+	+	ND	+	+	C1
b	C	-	+	ND	+	+	C2
c	C	-	-	+	+	+	C3
d	C	-	-	+	+	-	C4
e	C	-	-	-	ND	ND	Reject C packages (C5)
7c	S	++	+-/-	ND	ND	ND	Reject S packages
a	C	+	+	+	ND	ND	Save C packages
b	C	+	+	-	ND	ND	Reject bottom C packages
c	C	+	-	-	ND	ND	C5

TABLE 5. (Continued) Scenarios of temperature readings (TRs) of selected portion of a food lot of thick packages and subsequent decisions using the long form strategy^a

Scenarios ^b	Side(S) Core(C)	TR1	TR2	TR3	TR4	TR5	Decisions
8 ^a	S	++	+/-	ND	ND	ND	Reject S packages
a	C	+	+	ND	ND	ND	Save C packages
b	C	+	-	ND	ND	ND	Reject C packages

^a"++" indicates that two samples for TRs did not exceed acceptable temperature limit; "+/-" indicates that a single TR result is above the temperature limit and that if the first TR result is above the temperature limit, a second TR is unnecessary.

^bScenarios 1-5 are applicable to lots consisting of three or more layers. Scenarios 1, 3 and 4 are applicable to two-layered lots; Scenarios 1 and 3 are applicable to one-layered lots (underscored).

^cApplicable only to two-layered lots.

^dApplicable only to one-layered lots.

ND means not determined

TABLE 6. Scenarios of Temperature Readings of Selected Sampling Sites in food Lots of Thin Packages and Corresponding Decisions (Long Form Strategy)^a

Scenarios	Side(S) Core(C)	TR1	TR2	TR3	TR4	TR5	Decision
1	S	++	ND(++) ^a	ND(++) ^b	ND(++) ^c	++	Save all S packages
	C	ND	ND	ND	ND	ND	Save all C packages
2	S	++	ND(+/-) ^a (++) ^c	ND(+/-) ^b (++) ^d	ND(+/-) ^c	+/-	Reject bottom S packages
a	C	ND	ND(+) ^a	ND(+) ^b	ND(+) ^c	+	C1
b	C	+	ND(-) ^a	ND(-) ^b	+(-) ^c	-	Reject bottom C packages
3	S	+/-	++	ND(++) ^b	ND(++) ^c	++	Reject top S packages
a	C	+	ND	ND	ND	+	C1
b	C	-	ND	ND	ND	ND	Reject top C packages
4	S	+/-	+/-	++	ND	++	Reject top two S packages
a	C	+	+	ND	ND	ND	C1
b	C	-	+	ND	ND	ND	C2
c	C	-	-	ND	ND	ND	Reject top two C packages (C3)

TABLE 6. (Continued) Scenarios of Temperature Readings of Selected Sampling Sites in Food Lots of Thin Packages and Corresponding Decisions (Long Form Strategy)^a

Scenarios	Side(S) Core(C)	TR1	TR2	TR3	TR4	TR5	Decision
5	S	+/-/-	+/-/-	++	++(+/-1-)=	+/-/-	Rejects top two S packages and bottom S packages
a	C	+	ND	ND	ND	+	C1
b	C	-	+	ND	ND	+	C2
c	C	-	-	ND	ND	+	C3
d	C	-	-	ND	ND	-	Reject top two C packages and bottom C Packages
6	S	+/-/-	+/-/-	+/-/-	ND	ND	Reject all S packages
a	C	+	ND	ND	ND	+	C1
b	C	-	+	ND	ND	+	C2
c	C	-	-	+	ND	+	C3
d	C	-	-	-	ND	ND	Reject all C packages

^aApplicable only to two-layered lots.

^bApplicable only to three-layered lots.

^cApplicable only to four-layered lots.

ND means Not determined

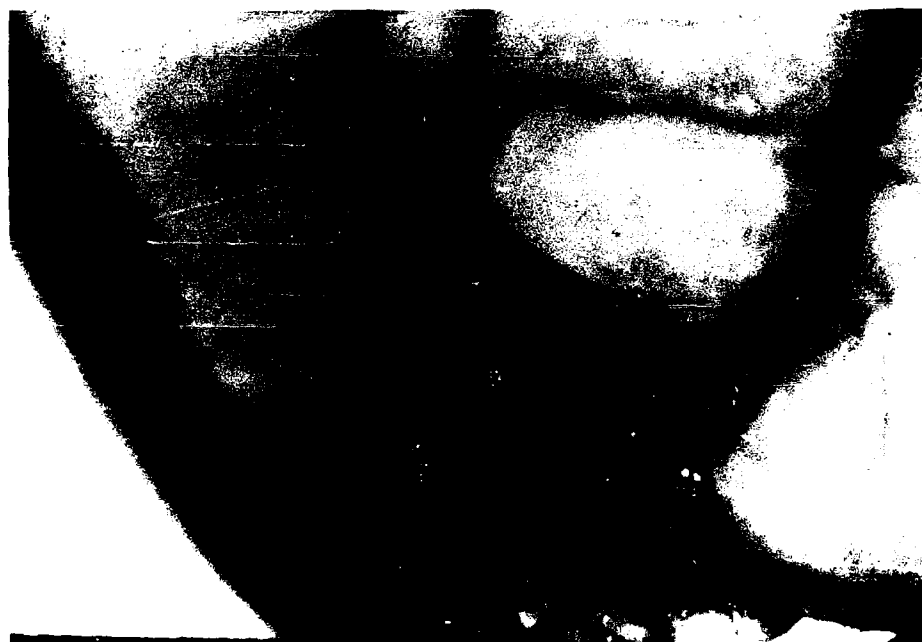


Plate 3. DISSECTION OF EPIPHYSEAL GROWTH PLATE CARTILAGE.

Sharp dissection was used to remove the growth plate cartilage from each of the three articulating surfaces. Although the thickness varied from animal to animal, care was taken to avoid areas of cartilage that appeared calcified. With this technique, the majority of the cartilage obtained, represented the resting, hypertrophic, and proliferative stages.

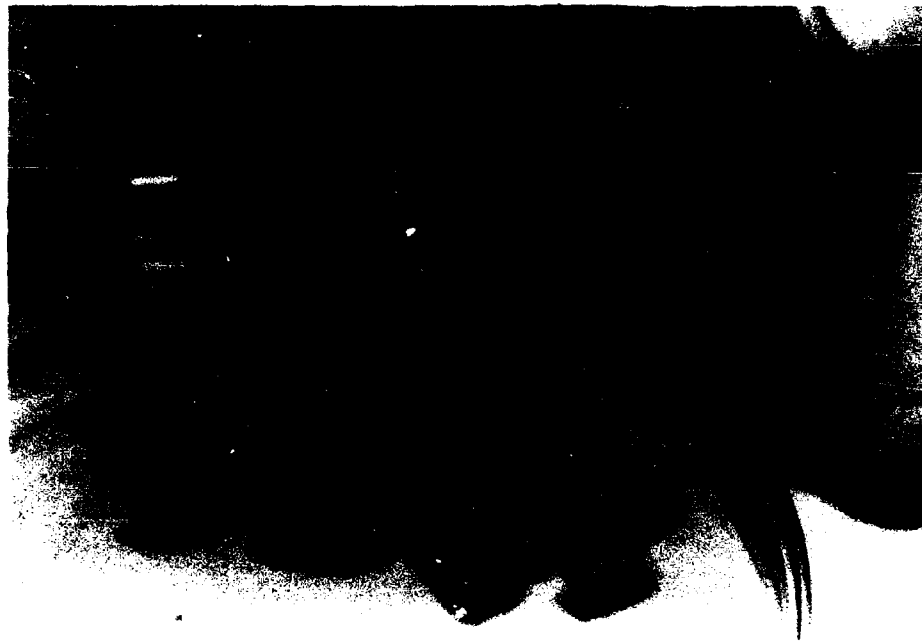


FIGURE 1. ^{14}C -DCCD BINDING AFFINITY FOR POLYPROPYLENE PLASTIC.

Time 0 is the control; solution was never placed in plastic. Time in minutes is the amount of time that sample solutions were exposed to plastic. In order to determine if DCCD binding sites in the plastic vials became saturated, one sample, after incubation for 30 minutes, was transferred to a clean plastic vial for an additional 30 minutes, resulting in a total of 60 minutes* that excess DCCD was exposed to plastic. In a similar manner, another sample was exposed to plastic for a total of 90 minutes**; 30 minutes in each of three successive plastic vials.

ANOVA revealed that compared to the control all samples had a significant reduction in ^{14}C -DCCD at $P < .005$. There was not a significant difference among samples placed in one plastic tube at any time ranging from 5 to 30 minutes ($p=0.789$). However, transferring the samples to fresh plastic vials resulted in further statistically significant reductions in ^{14}C -DCCD ($P < .005$).

DCCD Plastic Binding

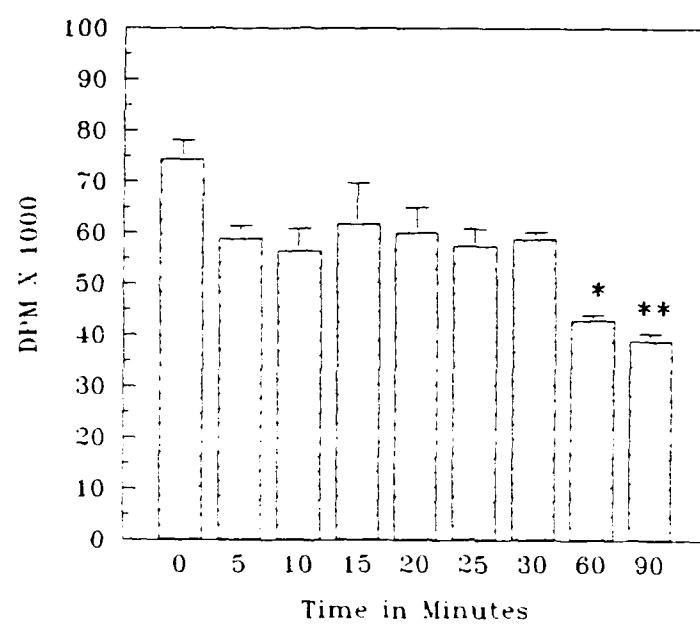


Plate 4. AUTORADIOGRAPH DEMONSTRATING EFFECTS OF ^{14}C -DCCD
PLASTIC BINDING.

Lane 1 is the autoradiographic representation of molecular weight markers, previously silver stained, that were labeled with a light emitting ink. Molecular weight markers (Bethesda Research Laboratories #6000 SA) are: Insulin (A & B chain) 2,300 and 3,400; Bovine trypsin inhibitor 6,200; Lysozyme 14,300; B-Lactoglobulin 18,400; α -Chymotrypsinogen 25,700; and Ovalbumin 43,000.

Samples of acid proteolipid extract were incubated with ^{14}C -DCCD for 30 minutes. Lane 2 demonstrates excess radiolabel at the gel front when plastic binding is not used. Lane 3 demonstrates that significant amounts of ^{14}C -DCCD are removed when the samples are exposed to plastic for 30 minutes prior to analysis by SDS-PAGE.

	1	2	3
Mr x 10 ³			
43-	•		
26-	•		
18-	•		
14-	•		
6-	•		
2-	•		



III. RESULTS

A. Sample Yields.

The epiphyses of the proximal portion of each metatarsus yielded, on average: 212 ± 21 mg growth plate cartilage (wet weight), 657 ± 45 ug of matrix vesicle preparation (lyophilized weight), 39 ± 11 ug of matrix vesicle protein, 45 ± 11 ug of crude proteolipid and phospholipid, 1.25 ± 0.4 ug of neutral proteolipid extract protein, and 0.85 ± 0.3 ug of acid proteolipid extract protein. Calculations from this data show that proteolipid protein accounts for $5.3\% \pm 1.2\%$ of total matrix vesicle protein (3.1% from neutral and 2.2% from acid proteolipid extract). Total proteolipid protein accounts for $3.6\% \pm 0.5\%$ of total proteolipid extract weight (1.6% from neutral and 2.44% from acid proteolipid extract).

B. Electron Microscopy.

Plate 5 is a representative example of the appearance of the matrix vesicle enriched preparation with transmission electron microscopy. These micrographs show large numbers of membrane bound structures ranging in size from 0.01 to 0.25 microns. There is no evidence of mineralization associated with these structures.

C. Enzyme Analyses.

Figures 2-4 illustrate the relative enzyme activities of matrix vesicles compared to the cell plasma membrane preparation. Specific activity of matrix vesicle alkaline phosphatase was 10.45 $\mu\text{moles P}_i/\text{ug protein/minute}$ whereas plasma membrane specific activity was 5.05 $\mu\text{moles P}_i/\text{ug protein/minute}$. Although the absolute enzyme activities varied from preparation to preparation, specific activity of alkaline phosphatase was enriched two-fold in the matrix vesicles compared to that of the plasma membrane. Matrix vesicle 5'-nucleotidase activity was 595 DPM/ug protein/minute and 364 DPM/ug protein/minute in the cell plasma membranes. This enzyme was consistently enriched in the matrix vesicle fraction. In contrast, ouabain sensitive Na^+/K^+ ATPase activity was greatest in the plasma membrane. Matrix vesicle enzyme activity was 9.16 pmoles $\text{P}_i/\text{ug protein/minute}$ versus 18.97 pmoles $\text{P}_i/\text{ug protein/minute}$. ATPase inhibition by ouabain ranged from 11%-22% depending on the preparation. However, within each preparation, the % inhibition was the same for membranes and matrix vesicles (Figure 5).

D. Isolation of Matrix Vesicle Proteolipid.

Plate 6 illustrates typical profiles of proteolipid elution on a Sephadex LH-20 column. Absorbance readings at 280 nm indicate that the sample was collected immediately after the void volume beginning at fraction 21. Material

continued to elute from the column until fraction 50, but it is clear that the majority of the sample was collected in fractions 21-29 which is the void volume peak.

E. Gel Electrophoresis.

1. Silver staining patterns.

Analysis of crude proteolipid by SDS-PAGE (Plate 7) demonstrated heavy silver staining patterns with predominant bands at 2,000-3,000 and 10,000-18,000 M_r for the acid proteolipid extract (lane 2) and at 2,000-6,000 M_r for the neutral proteolipid extract (lane 3). Partially delipidated proteolipids are depicted in plate 8. Broad irregular silver stained bands are located at M_r 3,000-8,000 and 16,000-21,000 for the proteolipid extracted with $CHCl_3:CH_3OH$ at 2:1 (v/v) (lane 2) and M_r 3,000-6,000 and 14,000-16,000 (lane 3) for the proteolipid extracted with $CHCl_3:CH_3OH:HCl(conc)$ at 200:100:1. Bands were generally wide with a characteristic "W" shape, especially evident in the neutral proteolipid extract. In addition, negative staining material was also present in the gels. This becomes even more apparent when the staining patterns are compared to the position of radioactive labelling on gels (Plate 9).

2. ^{14}C -DCCD binding characteristics.

Proteolipids isolated by neutral or acidified solvents exhibited specific binding to ^{14}C -DCCD. Plate 9

demonstrates that label is associated with the silver staining protein (M_r 3,000-5,000) present in the acidified solvent extract. No label was associated with the protein band at M_r 14,000-16,000 (lanes 2 and 3). Although no protein bands were detectable after silver staining in lane 4 containing neutral proteolipid extract, the fluorogram of this gel, lane 5 did demonstrate the presence of material to which ^{14}C -DCCD had bound at M_r 3,500-6,000 and at the origin. Binding present in both proteolipid extracts was observed to be directly proportional to the concentration of ^{14}C -DCCD incubated with the sample (Plate 10).

The results of competitive binding studies are depicted in Plate 11. Treatment versus control ratios were calculated from densitometric scans of these fluorograms to quantitate the inhibition of ^{14}C -DCCD binding. Unlabeled DCCD decreased binding of labeled ^{14}C -DCCD to both neutral and acid proteolipid extracts in a linear fashion. In a similar fashion, the nucleophile glycine ethyl ester (GEE) reduced the binding of ^{14}C -DCCD to both neutral and acid proteolipid extracts. ^{14}C -DCCD binding to either neutral or acid proteolipid was not affected by competitive binding with the carbodiimide EDAC.

Plate 5. TRANSMISSION ELECTRON MICROGRAPH OF MATRIX
VESICLE PREPARATION.

Representative TEM of matrix vesicle preparation.
Magnification X 40,000. Vesicles range in size from 100 A
to 2500 A. There is no evidence of calcification.

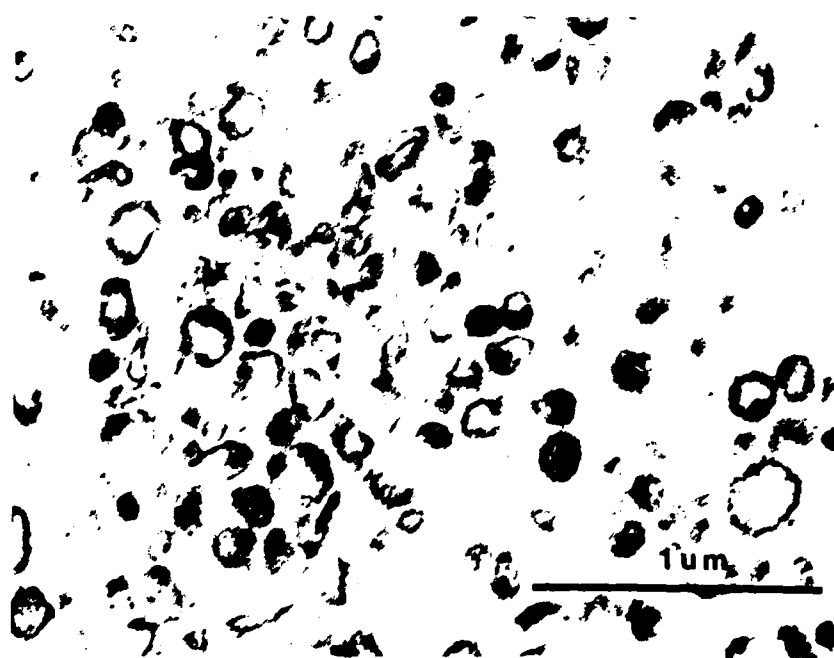


FIGURE 2. ALKALINE PHOSPHATASE ACTIVITY.

Epiphyseal growth plate cartilage from the proximal portion of 49-52 day old broiler strain chickens was digested in collagenase for 15 hours. Plasma membranes and matrix vesicles were separated by differential centrifugation. The specific activity of alkaline phosphatase was determined as umoles of phosphate hydrolyzed from p-nitrophenol/mg Lowry protein/minute. Data from two preparations are presented as average values \pm S.D.

Alkaline Phosphatase Activity

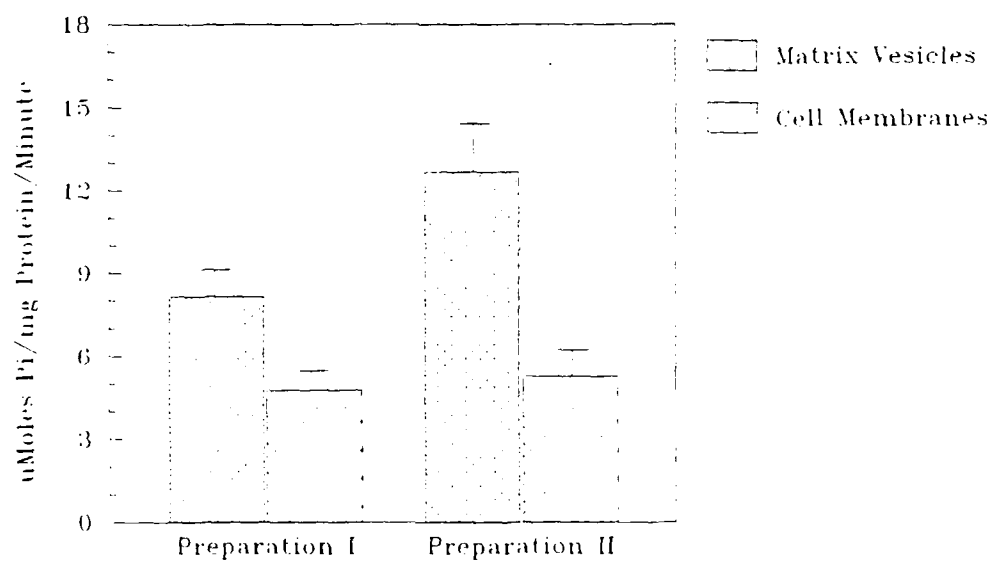


FIGURE 3. 5'-NUCLEOTIDASE ACTIVITY.

Epiphyseal growth plate cartilage from the proximal portion of 49-52 day old broiler strain chickens was digested in collagenase for 15 hours. Plasma membranes and matrix vesicles were separated by differential centrifugation. The specific activity of 5'-Nucleotidase was determined by expressing the cleavage of inorganic phosphate from cyclic AMP as disintegrations per minute (DPM)/ug Lowry protein/minute. Data from two preparations are presented as average values \pm S.D.

5'-Nucleotidase Activity

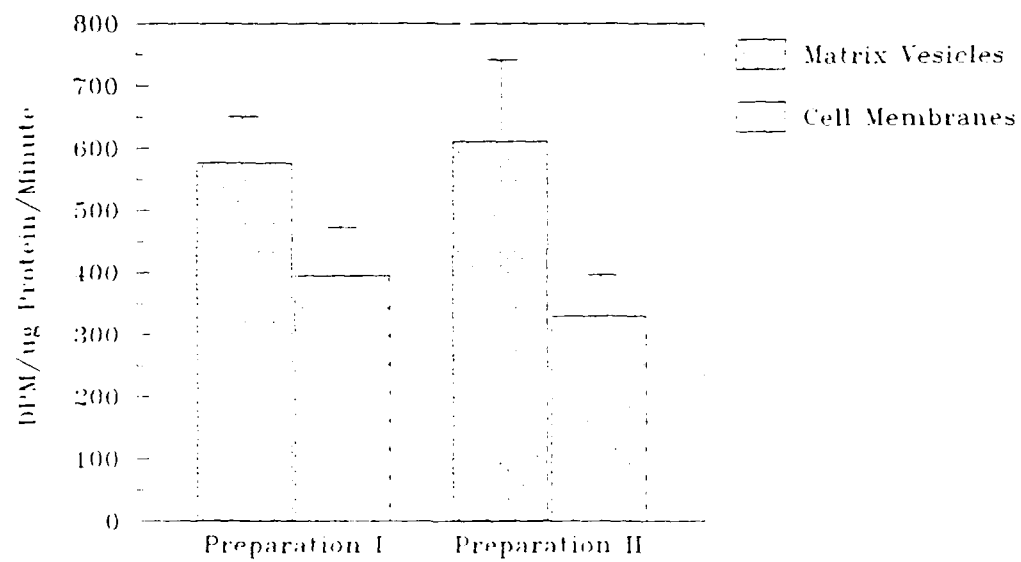


FIGURE 4. OUABAIN SENSITIVE Na^+/K^+ ATPASE ACTIVITY.

Epiphyseal growth plate cartilage from the proximal portion of 49-52 day old broiler strain chickens was digested in collagenase for 15 hours. Plasma membranes and matrix vesicles were separated by differential centrifugation. The specific activity of ouabain sensitive Na^+/K^+ ATPase was determined by measuring the amount of inorganic phosphate cleaved from ATP with and without ouabain in the reaction mixture. Data from two preparations are presented as average values \pm S.D.

Ouabain Sensitive $\text{Na}^+/\text{K}^+\text{ATPase}$ Activity

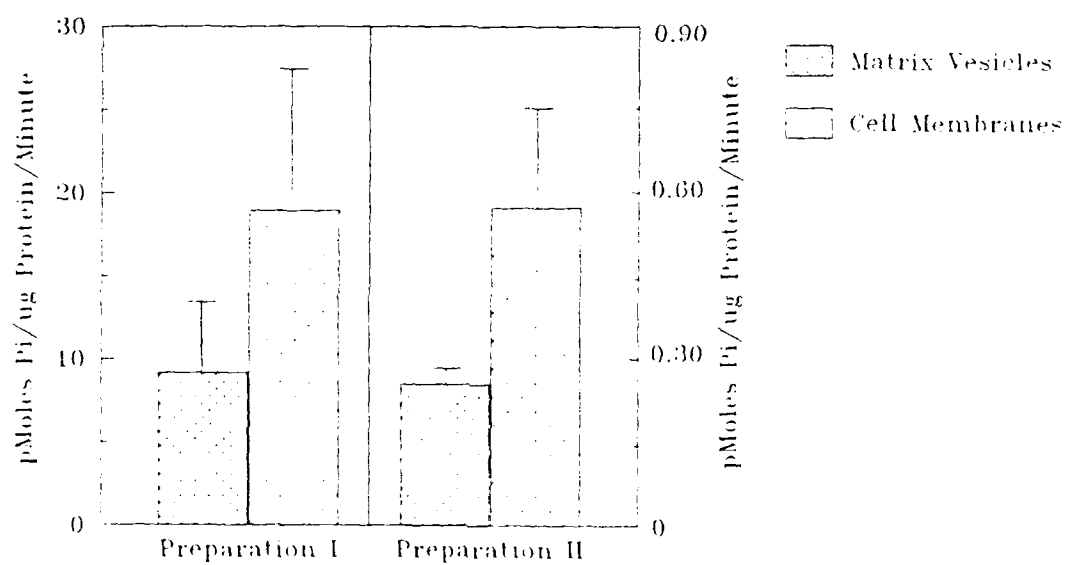


FIGURE 5. % INHIBITION OF OUABAIN SENSITIVE Na^+/K^+ ATPASE.

Epiphyseal growth plate cartilage from the proximal portion of 49-52 day old broiler strain chickens was digested in collagenase for 15 hours. Plasma membranes and matrix vesicles were separated by differential centrifugation. The % inhibition of ATPase activity by ouabain was determined by measuring the amount of inorganic phosphate cleaved from ATP with and without ouabain in the reaction mixture. Data from two preparations are presented as average values \pm S.D.

ATPase Inhibition by Ouabain

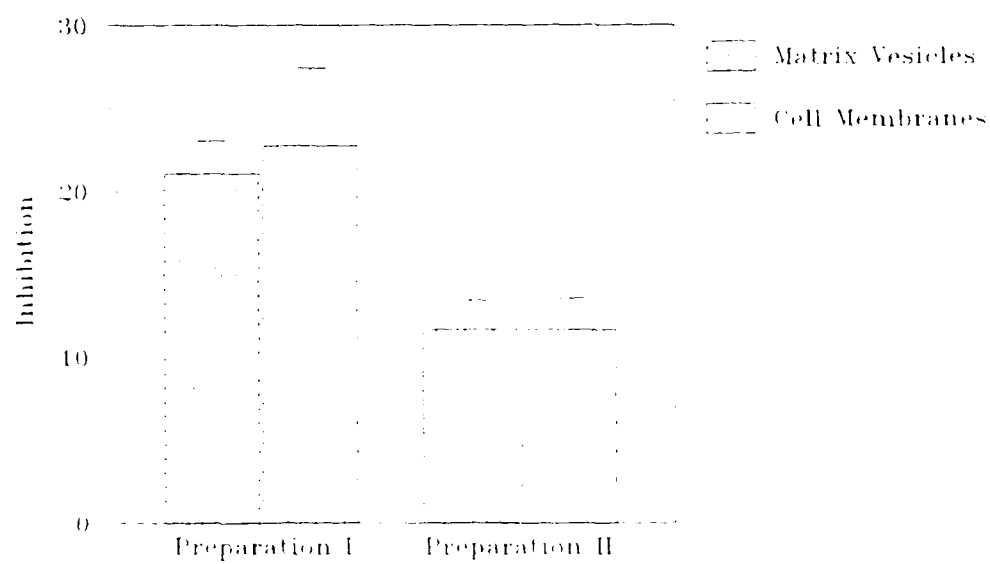


PLATE 6. HYDROXYPROPYLATED GEL FILTRATION (SEPHADEX LH-20)

Elution profiles of neutral chloroform:methanol extract (A) and acidified chloroform:methanol extract (B) from chick epiphyseal cartilage matrix vesicles.

Samples were applied to a 2.5 X 50 cm column equilibrated with 2:1 $\text{CHCl}_3:\text{CH}_3\text{OH}$. Eighty fractions of 4 ml each were collected and fractions 21-29 pooled as proteolipid. Eluates were monitored by absorption at 280 nm. Absorbance was plotted at 2 sensitivities in order to demonstrate greatest detail and to show the entire peak on the plot.

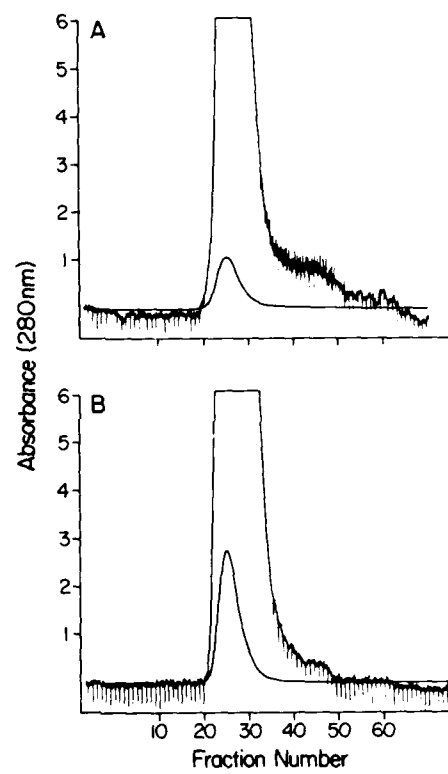


PLATE 7. SILVER STAINING PATTERNS OF CRUDE PROTEOLIPID
EXTRACT ON SDS-PAGE.

Lane 1: Molecular weight markers (Bethesda Research Laboratories #6000 SA) are: Insulin (A & B chain) 2,300 and 3,400; Bovine trypsin inhibitor 6,200; Lysozyme 14,300; B-Lactoglobulin 18,400; α -Chymotrypsinogen 25,700; and Ovalbumin 43,000.

Lane 2: Acid extract proteolipid.

Lane 3: Neutral extract proteolipid.

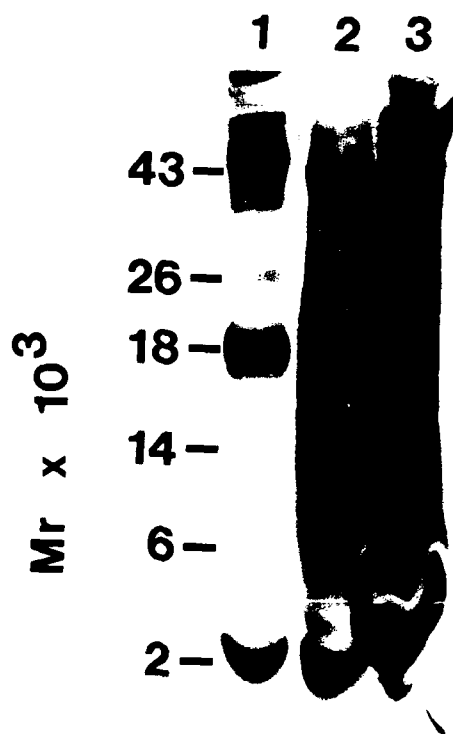


PLATE 8. SILVER STAINING PATTERNS OF PARTIALLY DELIPIDATED
PROTEOLIPIDS.

Lane 1: Molecular weight markers (Biorad Low Molecular weight standards): Lysozyme 14,400; Soybean Trypsin inhibitor 21,500; Carbonic anhydrase 31,000; Ovalbumin 45,000; Bovine serum albumin 66,200.

Lane 2: Neutral extract proteolipid.

Lane 3: Acid extract proteolipid.

Lane 4: Molecular weight markers (Sigma MW-SDS-17 kit) are Myoglobin (fragment III) 2,510; Myoglobin (fragment II) 6,210; Myoglobin (fragment I) 8,160; Myoglobin (fragment I + II) 14,400; and Myoglobin (polypeptide backbone) 16,960.

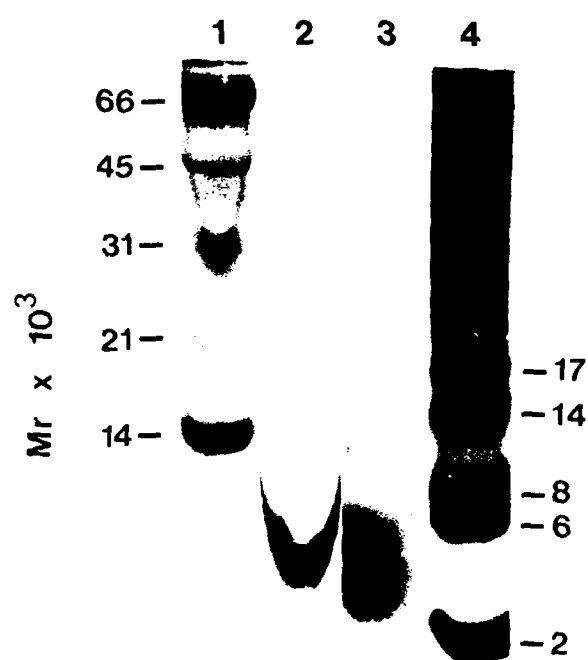


PLATE 9. SDS-PAGE WITH SILVER STAIN PATTERNS AND
ASSOCIATED ^{14}C -DCCD BINDING PATTERNS DETERMINED
BY AUTORADIOGRAPHY.

Lane 1: Molecular weight markers (Biorad low molecular weight standards) Lysozyme 14,400; Soybean trypsin inhibitor 21,500; Carbonic anhydrase 31,000; Ovalbumin 45,000; Bovine serum albumin 66,200; Phosphorylase B 92,500.

Lane 2: Silver stain of acid proteolipid extract.

Lane 3: Autoradiograph of lane 2.

Lane 4: Silver stain of neutral proteolipid extract.

Lane 5: Autoradiograph of lane 4.

Lane 6: Molecular weight markers Molecular weight markers (Bethesda Research Laboratories #6000 SA) are: Insulin (A & B chain) 2,300 and 3,400; Bovine trypsin inhibitor 6,200; Lysozyme 14,300; B-Lactoglobulin 18,400.

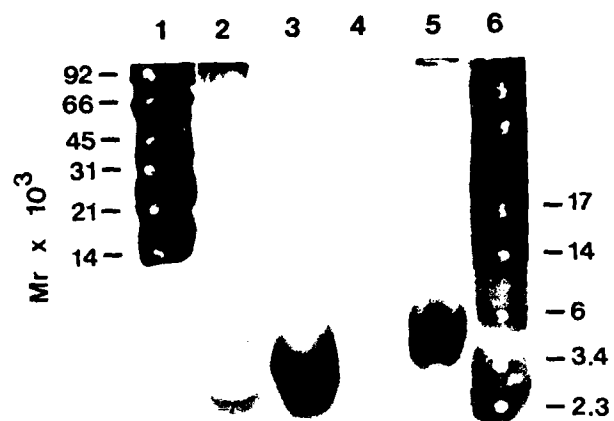
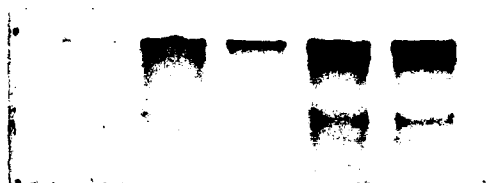


PLATE 10. FLUOROGRAM OF ^{14}C -DCCD LABELED CHICK MATRIX
VESICLE PROTEOLIPID.

Panel A, neutral proteolipid extract. Panel B, acidified solvent extract. Protein content of each lane was held constant at 10 ug. ^{14}C -DCCD was increased 2 X in concentration in each lane 1-5 respectively.

A




1 2 3 4 5

B





PLATE 11. FLUOROGRAMS OF CHICK MATRIX VESICLE PROTEOLIPID
INCUBATED WITH ^{14}C -DCCD IN THE PRESENCE OF
UNLABELED DCCD, EDAC OR GEE.


All samples contained the same initial concentration of ^{14}C -DCCD. Lane a, no competitor 1:0; lane b 1:1; lane c, 1:10; lane d, 1:100 and lane e, 1:1000. T/C are ratios of test/control as determined by densitometric scans. Column A represents acidified solvent extract, column B represents neutral solvent extract.


	A				
	a	b	c	d	e
DCCD					
T/C	1	.7	.5	.3	0

EDAC					
T/C	1	1	1	1	1

GEE					
T/C	1	.7	.5	.5	.1

	B				
	a	b	c	d	e
DCCD					
T/C	1	.5	.3	.1	0

EDAC					
T/C	1	.9	.9	.9	.9

GEE					
T/C	1	.5	.4	.3	.1

V. DISCUSSION

A. Sample Yields.

Total proteolipids extracted in this study accounted for 14.1% of matrix vesicle weight. This is substantially higher than the 2% reported by Boyan-Salyers et al. (1978). In the previous study proteolipids extracted with chloroform:methanol at 2:1 (v/v) and chloroform:methanol:HCl(conc) at 200:100:1 (v/v/v) were subjected to precipitation by addition of acetone with concomitant solubilization of neutral lipid. Boyan and Boskey (1984) have also used precipitation with diethyl ether as an alternative method for partial delipidation of smaller samples. Acid proteolipid extracts were not included. In the current investigation, neither acid nor neutral proteolipid extracts could be precipitated using either diethyl ether or acetone as described by Boyan and Clement-Cormier (1984). The reason for this is unclear, but may be because the sample size was insufficient to visualize a pellet. Consequently proteolipids used for SDS-PAGE and ^{14}C -DCCD binding may have contained a greater lipid component than those reported by Boyan Salyers (1978).

This conclusion is supported by the observations that neutral proteolipid extract protein accounts for 2.44% of total proteolipid weight and 3.14% of matrix vesicle protein. Corresponding yields for acid proteolipid extract

protein were 1.62% of proteolipid weight and 2.23% of total matrix vesicle protein. Considering the inability to delipidate these proteolipids, this data compares favorably with reports of proteolipid concentrations in a variety of tissues that range from 0.3% to 11% (Boyan-Salyers, 1981).

Yields per chicken foot dissected were only 1.23 ug of neutral proteolipid extract protein and 0.84 ug of acid proteolipid extract protein. These protein yields dictate that twenty to forty chicken feet must be dissected to prepare a sample for analysis on a single lane by SDS-PAGE.

B. Enzyme Analysis.

Analysis of the three plasma membrane enzyme markers: alkaline phosphatase, ouabain sensitive Na^+/K^+ ATPase, and 5'-nucleotidase, demonstrates that matrix vesicles differ from cell membranes. Alkaline phosphatase activity was enriched in matrix vesicles compared to cell membranes. This has been a consistent finding by numerous investigators in matrix vesicles from epiphyseal cartilage (Anderson, 1978; Majeska et al., 1975; Wuthier, 1982; Ali, 1983; and Caswell et al., 1987) and in matrix vesicles from cultured chondrocytes (Glaser and Conrad, 1981; Wuthier et al., 1985; Boyan et al., 1987; and Hale and Wuthier, 1987). Cell membranes were enriched in ouabain sensitive Na^+/K^+ ATPase by a factor of 2:1. Although two reports (Thyberg and Friberg, 1972; and Boyan et al., 1987) claimed that this enzyme was enriched in matrix vesicles, reports by Glaser

and Conrad (1981) and Majeska and Wuthier (1975) indicate that ouabain sensitive Na^+/K^+ ATPase is slightly elevated in the plasma membrane fraction. The discrepancy may be due to species differences since the former reports were based on rat cartilage whereas the latter reports including the current investigation utilized chicken cartilage tissue. The specific activity of 5' ribonucleotidase in matrix vesicles was double that found in cell membranes. This agrees with data reported by Boyan et al. (1987) indicating 5' ribonucleotidase activity in matrix vesicles of cultured chondrocytes ranged from 1.44 to 8 times higher than that found in cell membranes. This data indicates that both alkaline phosphatase and 5' nucleotidase can be used as enzyme markers for matrix vesicles.

While the ratios of each enzyme activity in matrix vesicles compared to cell membranes were consistent, there was wide variation in specific enzyme activity when comparing results of individual dissections. This became evident during the course of the investigation when it was necessary to change lots of Worthington Type II collagenase. Digestion of cartilage with collagenase from new lots resulted in several preparations which exhibited no activity for alkaline phosphatase, 5' nucleotidase, or ouabain sensitive Na^+/K^+ ATPase. Specific enzyme activity appeared to be related to the collagenase used in the digestion of the growth plate cartilage. In order to

confirm this, cell membrane preparations with an established baseline alkaline phosphatase activity were incubated with one of three collagenases that differed by either manufacturer or lot number. Results are shown in figure 6 and indicate that each collagenase diminished alkaline phosphatase activity by different amounts. It is hypothesized that protease impurities present in the collagenase are responsible for the degradation of enzyme activity. A wide range of specific enzyme activities in matrix vesicles have been reported in the literature. The reports of enzyme activity levels are based on: use of different collagenases for digestion, varying levels of sample purification, use of different assays, and expression of enzyme activities in dissimilar terms. Thus it is important to only compare specific enzyme activities between various fractions in a single preparation.

The enrichment of both alkaline phosphatase and 5'-nucleotidase in matrix vesicles lends support to the proposed model for membrane associated calcification. Both of these enzymes might play a role in degradation of mineralization inhibiting inorganic pyrophosphates within the confines of the matrix vesicle.

C. Characterization of Matrix Vesicle Proteolipid.

Analysis of matrix vesicle proteolipids by SDS-PAGE has proven to be difficult. While silver staining patterns are relatively constant, band resolution is poor, making

interpretation difficult. The presence of wide bands, the "W" shaped band associated with the neutral proteolipid extract, and areas with negative staining, are all attributed to a high phospholipid content which is likely to be an integral part of the proteolipid.

SDS-PAGE patterns of epiphyseal cartilage matrix vesicle proteolipids are similar to proteolipids extracted from cultured chondrocyte matrix vesicle proteolipids (Boyan et al., in press). Neutral proteolipid extracts from both exhibit broad silver staining bands at M_r 2,500 to 5,000. There are no reports in the literature that examine either acid proteolipid extracts or DCCD binding characteristics of proteolipids from vertebrate matrix vesicles.

Similarities and differences exist between proteolipids extracted from matrix vesicles and calcifying bacteria (Swain and Boyan, 1987). Based on silver staining, SDS-PAGE profiles of the neutral proteolipid extract from *Bacterionema matruchotti* demonstrates a broad "W" shaped band centered at M_r 10,000, while the neutral proteolipid extract from epiphyseal cartilage matrix vesicles has a similar shaped band centered at M_r 8,000. Acid proteolipid extracts from bacteria exhibited protein species at M_r 8,500; 18,000; 24,000; 38,000; 45,000; and 85,000. Similar extracts from matrix vesicles had staining bands at M_r 4,000-5,000 and 14,000-16,000. ^{14}C -DCCD only binds to the 8,500 M_r proteolipid species obtained by acid extraction of

the bacterial membrane, but for both acid and neutral proteolipid extracts of vertebrate matrix vesicles, binds at 5,000-6,000 M_r . For the neutral proteolipid extract this represents a protein species with a negative staining pattern; i.e., it did not stain with silver. The possibility exists that negatively stained bands do not contain protein, and that ^{14}C -DCCD binding is to phospholipids. However, since ^{14}C -DCCD would normally bind to phosphatidyl serine (PS) and since PS should migrate with the solvent front, this is probably not the case.

The fact that ^{14}C -DCCD binding is competitive with DCCD in a molar fashion (Fillingame, 1976)) indicates that the binding is specific for carboxyl groups. That EDAC, a hydrophilic carbodiimide, does not compete with ^{14}C -DCCD labeling indicates that the binding site resides within a hydrophobic portion of the proteolipid molecule (Cattell et al., 1971). The fact that GEE competes with the labeled ligand strongly suggests that the binding site is a carboxyl group, rather than a phenolic group of tyrosine (Schindler, 1982). It is important to note that these binding characteristics, although carried out in a TRIS buffer with 0.5% SDS, are similar to those described for known proteolipid ion channels (Siegel et al., 1983; Perlin et al., 1983), such as the F_o proteolipid channel of F_1/F_o ATPase.

Recent studies in this laboratory (Swain and Boyan, 1987) using *Bacterionema matruchotti* as a model of

calcification, have demonstrated ^{14}C -DCCD binding of the type described in this experiment. Further, reconstitution of the bacterial calcifiable proteolipid into proteoliposomes containing bacteriorhodopsin as a proton pump, demonstrated a specific ionophoric function for the calcifiable proteolipid (Boyan et al., 1985). It would be interesting to know if matrix vesicle proteolipid has ionophoric properties such as those demonstrated in the DCCD binding proteolipids of the calcifying bacterium. Such information would contribute to understanding the mechanisms of maintaining the critical ionic balances and pH (which are conducive to initial hydroxyapatite crystal formation) within the confines of the miniscule volume of the matrix vesicle. It is also possible that an antiport system of the type described by Brey and Rosen (1979) could exist whereby protons are exchanged for Ca^{+2} , thus maintaining pH at critical levels while importing Ca^{+2} to the mineralization site.

D. Removal of Excess DCCD from Samples.

Previous work with calcifiable proteolipid DCCD binding characteristics has relied on precipitation and centrifugation to remove unbound DCCD from the samples (Swain and Boyan, 1987). As already mentioned, proteolipid samples in this study could not be precipitated with either diethyl ether or acetone. Since this made it impossible to remove free ^{14}C -DCCD from the samples, the feasibility of binding

this excess ^{14}C -DCCD to plastic was studied. Under conditions used by other researchers, labeled DCCD is diluted with non-radioactive DCCD which results in precipitation of excess DCCD, both labeled and unlabeled (Renthal, personal communication). The disadvantages of this approach are the possibility of inaccurate analyses and displacement of already bound ^{14}C -DCCD. The results of the present study indicate that while not all solubilized ^{14}C -DCCD bound to the plastic vials, significant quantities were removed from solution.

The technique described utilizes only the inner sides of polypropylene tubes which present a minimal area. Since it is likely that ^{14}C -DCCD binding sites on this surface can become saturated, solutions were transferred to a second and a third polypropylene vial. A single transfer to a fresh polypropylene tube provided a further significant decrease in free ^{14}C -DCCD, but transfer to a third vial had no added effect. This implies that an equilibrium is reached at which point there is no further binding of ^{14}C -DCCD to plastic. Although this technique does not remove all ^{14}C -DCCD from solution, its value is evident when comparing SDS-PAGE autoradiographs. When plastic binding is not used, excess ^{14}C -DCCD accumulates from M_r 3,000 to the gel front. With plastic binding, label is seen only in association with the proteolipid to which it binds.

FIGURE 6. EFFECTS OF THREE DIFFERENT COLLAGENASES ON ALKALINE PHOSPHATASE ACTIVITY.

Plasma membrane and mitochondria suspension (obtained by digestion with 0.03% Worthington type II collagenase for 15 hours at 37°C) with an established alkaline phosphatase activity was incubated with three 0.03% collagenase solutions (TRIS-HCl buffer pH 7.4) for 1 hour at 37°C. Alkaline phosphatase activity (umoles P_i released/mg protein/minute) was measured for each sample to determine the effects of the collagenase solution on enzyme activity. Reduction of enzyme activity is expressed as the % degradation per hour of digestion compared to baseline activity levels.

COLLAGENASE TYPE	ALKALINE PHOSPHATASE ACTIVITY	% DEGRADATION PER HOUR OF DIGESTION
BASELINE ACTIVITY	16.2 \pm 0.4	na
WORTHINGTON 4176 LOT # 47S266 134 U/mg	11.8 \pm 0.5	27.4 \pm 3.1
COOPER BIOMEDICAL 4176 LOT # 44D813 132 U/mg	3.1 \pm 0.8	80.7 \pm 4.9
COOPER BIOMEDICAL 4196 LOT # 44K6380 166 U/mg	11.2 \pm 0.9	31.1 \pm 5.8

V. CONCLUSIONS

A. Matrix vesicle proteolipid yield from broiler strain chicken metatarsal bones is limited. With techniques currently available, the minute yields limit the practicality of using this model for investigation of vertebrate calcifying proteolipids.

B. Compared to cell plasma membranes matrix vesicles are enriched in alkaline phosphatase and 5'-nucleotidase, and deficient in ouabain sensitive Na^+/K^+ ATPase. It has been demonstrated that the specific activities of these enzymes are sensitive to the type of collagenase used in digestion of the cartilage. Alteration of enzyme activity in this step does not appear to alter the properties of matrix vesicle proteolipids examined in this research.

C. Vertebrate matrix vesicle proteolipid has similarities and differences compared to calcifying proteolipids obtained from a bacterial model. Both bind ^{14}C -DCCD in a concentration dependent fashion. GEE competes for these binding sites while EDAC does not. This implies that ^{14}C -DCCD binds to a carboxyl residue in a hydrophobic portion of the proteolipid. However, ^{14}C -DCCD binds to both acid and neutral proteolipid extract in the vertebrate model and only to acid proteolipid extract in the bacterial model. In addition, relative molecular weights determined by SDS-PAGE are different between the two

systems. A possible explanation for these differences is that the vertebrate proteolipids are more complex, with the proteins more tightly bound to their associated lipids.

D. Based on the similarities of the vertebrate and bacterial models, and the difficulties in working with the vertebrate model, future research on the role of proteolipids in mineralization should be based on either a bacterial or cell culture model.

E. The technique of binding excess ^{14}C -DCCD to plastic appears to be a useful method of removing excess radiolabel when dealing with samples that cannot be precipitated. It is likely that this technique could be refined to remove larger proportions of excess ^{14}C -DCCD.

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VITA

PII Redacted

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